(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number
WO 01/20011 A1

(51) International Patent Classification[7]: C12N 15/82, 9/02, 9/10, A01H 5/00, C12P 23/00 // (C12N 9/02, C12R 1:89)

(21) International Application Number: PCT/SE00/01767

(22) International Filing Date: 13 September 2000 (13.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9903336-7 17 September 1999 (17.09.1999) SE

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/20011 A1

(54) Title: DNA CONSTRUCT AND ITS USE

(57) Abstract: A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region is disclosed. The DNA construct may additionally comprise a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant. The peptide with enzyme activity is preferably a peptide with β-carotene C-4-oxygenase activity, e.g. from the alga <*aematococcus pluvialis*. Comprised by the invention are also a transgenic oilseed plant cell, e.g. of rape, sunflower, soybean or mustard origin, and a transgenic oilseed plant-produced xanthophyll, such as canthaxanthin or astaxanthin, and also astaxanthin esters.

DNA construct and its use.

The present invention relates to a new DNA construct for transformation into oilseed plants. The DNA construct comprises nucleotide sequences encoding peptides with enzyme activities necessary for the high-level production and esterification of keto group-containing xanthophylls in oilseed plants.

Background of the invention

Carotenoids are produced *de novo* by plants, fungi, algae and some bacteria. A number of biosynthetic steps are needed for the biological production of the carotenoids. There are two chemically different groups of carotenoids, namely carotenes containing only carbon and hydrogen molecules and xanthophylls containing oxygen in the molecule in addition to carbon and hydrogen.

The xanthophylls, and particularly astaxanthin (3,3'-dihydroxy-β-β-carotene-4,4'-dione), are often colored pigments and are used as such or as anti-oxidants.

Carotenes are biological precursors for the production of the oxygen-containing xanthophylls. There are two types of enzymes responsible for the introduction of hydroxy groups and keto groups into the carotenes, namely hydroxylases and ketolases, respectively.

The keto group-containing xanthophyll astaxanthin, which has keto and hydroxy groups, is biosynthetically produced from beta-carotene.

Large-scale production of xanthophylles from natural sources is at present performed by AstaCarotene AB, Gustavsberg, Sweden, by cultivation of the alga *Haematococcus pluvialis* for the production of astaxanthin in esterified form.

It would be desirable to be able to produce keto group-containing xanthophylls particularly astaxanthin, in oilseed plants. Oilseed plants have naturally β-carotene hydroxylases but lack β-carotene C-4-oxygenase enzymes or ketolases.

Description of the invention

The present invention provides DNA constructs enabling and promoting production of keto group containing xanthophylls, especially astaxanthin, in oilseed plants, such as rape, sunflower, soybean and mustard. The DNA construct is transformed into the oilseed plant cell for expression of a protein or fused protein which has an enzyme activity enabling keto group insertion into a carotene or hydroxy carotene for the biosynthetic production of a keto group containing xanthophyll, such as cantaxanthin (β,β-carotene-4,4'-dione) and/or astaxanthin. Use is thus made of the biosynthetic pathway of the oilseed plant to

produce carotenoids. The naturally occurring synthesis of carotenoids involves a number of enzymes, namely 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β-carotene hydroxylase, and β-carotene C-4-oxygenase. Genes coding for peptides having these enzymatic activities may be inserted into the DNA construct of the invention, one or several per construct, to promote high-level production in the transgenic oilseed plant. In case only one enzyme coding gene is inserted per plant, two or more plants may be sexually interbred to produce plants containing all the desired enzyme activities.

Thus, the present invention is directed to a DNA construct comprising in the 5′ to 3′ direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

In a preferred embodiment of the invention the DNA construct additionally comprises between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant.

The DNA construct is preferably such that the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production is selected from the group consisting of peptides with 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β-carotene hydroxylase, and β-carotene C-4-oxygenase activity. To promote esterification of astaxanthin a nucleotide sequence coding for a peptide with acyl transferase activity may be included in the group.

In a preferred embodiment of the DNA construct according to the invention the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence coding for a N-terminally truncated β-carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.

An example of the DNA construct of the invention is presented in the sequence listing as SEQ ID NO:1 and in Fig.1.

The present invention is also directed to a transgenic oilseed plant cell comprising the DNA construct of the invention, and preferably the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

The invention is additionally directed to transgenic oilseed plant-produced xanthophyll, e.g. canthaxanthin and astaxanthin.

A preferred aspect of the invention is directed to transgenic oilseed plant-produced astaxanthin esters.

The present invention will now be illustrated with reference to the DNA construct disclosed in the sequence listing and in Fig.1, and the following description of embodiments. However, the invention is not limited to these exemplifications.

Short description of the drawings

Fig.1 illustrates the nucleotide sequence of the DNA construct comprising the napin promoter, the chloroplast localization signal, the N-terminally truncated β-carotene C-4-oxygenase gene and the termination sequence, and the deduced amino acid sequences of the transit peptide and the β-carotene C-4-oxygenase.

Description of embodiments

The invention is illustrated by production of astaxanthin in the seed of oilseed rape. The astaxanthin produced in the seed of the transgenic plant is extracted as part of the extracted oil. By use of conventionally used protocols for Agrobacterium tumefaciens mediated transformation such as described by (Hoekema et al.1983, An et al. 1986, Fry et al. 1987, DeBlock et al. 1988, Radke et al.1988, or Moloney et al. 1989) transgenic plants are produced having a chimeric DNA construct that is genetically inherited and is able to produce astaxanthin. The nucleotide sequence of the chimeric DNA construct consist of four parts of different genetic origin namely: (1) a promoter, (2) a localization signal, (3) a β-carotene C-4-oxygenase coding region and (4) a termination sequence.

The napin promoter directs transcription to the seed of oilseed rape (Stålberg et al 1996). This promoter was coupled to a localization signal similar but not identical to a transit peptide (TP) of Rbcs1a (Krebbers, 1988) that directs the translated product of a fused gene to the chloroplast. The promoter and the TP sequence were ligated to a part of the coding sequence of a ketolase gene BCK (Kajiwara et al. 1995). This enzyme oxygenates β-carotene to canthaxanthin, (Fraser et al. 1997). The chimeric DNA construct was then coupled to a suitable termination sequence, e.g. that of the Agrobacterium tumefaciens nopaline synthase gene (the nos 3′ end)(Bevan et al. 1983), as illustrated in Fig.1.

<u>Cellular storage of Astaxantin</u>

The storage of large amounts of free astaxanthin in plants will be difficult due to toxic effects of the molecule as it intercalates in the plant membranes. An effective esterification of astaxanthin to fatty acids enables storage of the esterified molecules in triacylglycerol containing oleosomes. Thus, an acyl transferase can be claimed to be of fundamental importance for the process, as is proteins that can mediate transport of different forms of astaxanthin from the chloroplast to the vesicles.

<u>Sequences and oligonucleotides used in the construction of the DNA construct</u>

1. Napin promoter (GeneBank ACCESSION No. J02798)

This promoter sequence, a 1145 base pair fragment including the 5' leader sequence has a unique HindIII site at the 5' end. The 3' end was synthesized with an additionally 6 nucleotide BamHI site.

2. Transit peptide similar to RBCS1a (GeneBank ACCESSION No. X13611, X14565)

The transit peptide (TP) was amplified by PCR from –28 to the end of the transit cleavage aa=54/55 site of the Rbcs1a gene. The 5' end was synthesized with a BamHI site and similarly the 3' sequence was synthesized with a XbaI site. The two following oligonucleotides were used for the PCR amplification.

BamHI

5' primer: TP1 5´AGAC **GGATCC** TCAGTCACACAAAGAGTA 3´

SacI XbaI

3' primer: TP2 5´GTTC **GAGCTC** **TCTAGA** CATGCAGTTAACGC 3´

3. BCK (β-carotene C-4 oxygenase) (Genebank ACCESSION No. D45881)

The BCK fragment was amplified by PCR including a 5' XbaI site and was ligated to the TP already described. The 5' primer (BCK1) used for PCR, is homologous to the BCK sequence from nucleotide 264 and the 3' oligonucleotide (Ax40) ends with a stop codon and was synthesized with a SacI restriction site for cloning. The synthesized fragment was fused to the TP as shown in Fig 1.

Oligonucleotides used for PCR:

XbaI

5´ primer: BCK1 5´ACAG **TCTAGA** ATGCCATCCGAGTCGTCA 3´

SacI

3´primer: AX40 5´CACC**GAGCTC**CATGACACTCTTGTGCAGA 3´

Description of SEQ ID NO:1 and SEQ ID NO:2

The sequences shown i Fig.1 are the same as the two sequences which are shown in the sequence listing.

The SEQ ID NO:1 is a nucleotide sequence composed of the following features:

	Nucleotide No.
Cloning site HindIII	1-6
Napin Promoter	1-1145
Cloning site BamHI	1146-1151
Transit peptide leader	1152-1178
Transit peptide coding	1179-1347
Cloning site XbaI	1348-1353
β-carotene C-4-oxygenase	1354-2217
β-carotene C-4-oxygense 3' untranslated	2218-2266
Cloning site SacI	2267-2272
Nopaline synthetase termination	2273-2536
Cloning site EcoRI	2538-2543

The SEQ ID NO: 2 is a deduced amino acid sequence of the fusion protein of the transit peptide and the peptide with β-carotene C-4-oxygenase activity.

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Claims

1. A DNA construct comprising in the 5′ to 3′ direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

2. The DNA construct according to claim 1, which between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity additionally comprises a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant.

3. The DNA construct according to claim 1 or 2, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β-carotene hydroxylase, β-carotene C-4-oxygenase, and acyl transferase activity.

4. The DNA construct according to any one of claims 1 - 3, wherein the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence coding for a N-terminally truncated β-carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.

5. The DNA construct according to claim 4, wherein the nucleotide sequence is SEQ ID NO:1.

6. Transgenic oilseed plant cell comprising the DNA construct of any one of claims 1-5 .

7. Transgenic oilseed plant cell according to claim 6, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

8. Transgenic oilseed plant-produced xanthophyll.

9. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein the xanthophyll is canthaxanthin

10. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein the xanthophyll is astaxanthin.

11. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein the xanthophyll is astaxanthin esters.

Napin promoter
AAGCTTTCTTCATCGGTGATTGATTCCTTTAAAGACTTATGTTTCTTATCTTGCTTCTGA

GGCAAGTATTCAGTTACCAGTTACCACTTATATTCTGGACTTTCTGACTGCATCCTCATT

TTTCCAACATTTTAAATTTCACTATTGGCTGAATGCTTCTTCTTTGAGGAAGAAACAATT

CAGATGGCAGAAATGTATCAACCAATGCATATATACAAATGTACCTCTTGTTCTCAAAAC

ATCTATCGGATGGTTCCATTTGCTTTGTCATCCAATTAGTGACTACTTTATATTATTCAC

TCCTCTTTATTACTATTTTCATGCGAGGTTGCCATGTACATTATATTTGTAAGGATTGAC

GCTATTGAGCGTTTTTCTTCAATTTTCTTTATTTTAGACATGGGTATGAAATGTGTGTTA

GAGTTGGGTTGAATGAGATATACGTTCAAGTGAAGTGGCATACCGTTCTCGAGTAAGGAT

GACCTACCCATTCTTGAGACAAATGTTACATTTTAGTATCAGAGTAAAATGTGTACCTAT

AACTCAAATTCGATTGACATGTATCCATTCAACATAAAATTAAACCAGCCTGCACCTGCA

TCCACATTTCAAGTATTTTCAAACCGTTCGGCTCCTATCCACCGGGTGTAACAAGACGGA

TTCCGAATTTGGAAGATTTTGACTCAAATTCCCAATTTATATTGACCGTGACTAAATCAA

CTTTAACTTCTATAATTCTGATTAAGCTCCCAATTTATATTCCCAACGGCACTACCTCCA

AAATTTATAGACTCTCATCCCCTTTTAAACCAACTTAGTAAACGTTTTTTTTTTTAATTT

TATGAAGTTAAGTTTTTACCTTGTTTTTAAAAAGAATCGTTCATAAGATGCCATGCCAGA

ACATTAGCTACACGTTACACATAGCATGCAGCCGCGGAGAATTGTTTTTCTTCGCCACTT

GTCACTCCCTTCAAACACCTAAGAGCTTCTCTCTCACAGCACACACATACAATCACATGC

GTGCATGCATTATTACACGTGATCGCCATGCAAATCTCCTTTATAGCCTATAAATTAACT

CATCCGCTTCACTCTTTACTCAAACCAAAACTCATCAATACAAACAAGATTAAAAACATA

```
 End      -28 untranslated leader      TP start
CACGAGGATCCTCAGTCACACAAAGAGTAAAGAAGAACAATGGCTTCCTCTATGCTCTCT
                                         M  A  S  S  M  L  S

TCCGCTACTATGGTTGCCTCTCCGGCTCAGGCCACTATGGTCGCTCCTTTCAACGGACTT
 S  A  T  M  V  A  S  P  A  Q  A  T  M  V  A  P  F  N  G  L

AAGTCCTCCGCTGCCTTCCCAGCCACCCGCAAGGCTAACAACGACATTACTTCCATCACA
 K  S  S  A  A  F  P  A  T  R  K  A  N  N  D  I  T  S  I  T
```

FIG.1

```
                              TP End      C-4-Oxygenase
AGCAACGGCGGACGCGTTAACTGCATGTCTAGAATGCCATCCGAGTCGTCAGACGCAGCT
 S  N  G  G  R  V  N  C  M  S  R  M  P  S  E  S  S  D  A  A

CGTCCTGCGCTAAAGCACGCCTACAAACCTCCAGCATCTGACGCCAAGGGCATCACGATG
 R  P  A  L  K  H  A  Y  K  P  P  A  S  D  A  K  G  I  T  M

GCGCTGACCATCATTGGCACCTGGACCGCAGTGTTTTTACACGCAATATTTCAAATCAGG
 A  L  T  I  I  G  T  W  T  A  V  F  L  H  A  I  F  Q  I  R

CTACCGACATCCATGGACCAGCTTCACTGGTTGCCTGTGTCCGAAGCCACAGCCCAGCTT
 L  P  T  S  M  D  Q  L  H  W  L  P  V  S  E  A  T  A  Q  L

TTGGGCGGAAGCAGCAGCCTACTGCACATCGCTGCAGTCTTCATTGTACTTGAGTTCCTG
 L  G  G  S  S  S  L  L  H  I  A  A  V  F  I  V  L  E  F  L

TACACTGGTCTATTCATCACCACACATGACGCAATGCATGGCACCATAGCTTTGAGGCAC
 Y  T  G  L  F  I  T  T  H  D  A  M  H  G  T  I  A  L  R  H

AGGCAGCTCAATGATCTCCTTGGCAACATCTGCATATCACTGTACGCCTGGTTTGACTAC
 R  Q  L  N  D  L  L  G  N  I  C  I  S  L  Y  A  W  F  D  Y

AGCATGCTGCATCGCAAGCACTGGGAGCACCACAACCATACTGGCGAAGTGGGGAAAGAC
 S  M  L  H  R  K  H  W  E  H  H  N  H  T  G  E  V  G  K  D

CCTGACTTCCACAAGGGAAATCCCGGCCTTGTCCCCTGGTTCGCCAGCTTCATGTCCAGC
 P  D  F  H  K  G  N  P  G  L  V  P  W  F  A  S  F  M  S  S

TACATGTCCCTGTGGCAGTTTGCCCGGCTGGCATGGTGGGCAGTGGTGATGCAAATGCTG
 Y  M  S  L  W  Q  F  A  R  L  A  W  W  A  V  V  M  Q  M  L

GGGGCGCCCATGGCAAATCTCCTAGTCTTCATGGCTGCAGCCCCAATCTTGTCAGCATTC
 G  A  P  M  A  N  L  L  V  F  M  A  A  A  P  I  L  S  A  F

CGCCTCTTCTACTTCGGCACTTACCTGCCACACAAGCCTGAGCCAGGCCCTGCAGCAGGC
 R  L  F  Y  F  G  T  Y  L  P  H  K  P  E  P  G  P  A  A  G

TCTCAGGTGATGGCCTGGTTCAGGGCCAAGACAAGTGAGGCATCTGATGTGATGAGTTTC
 S  Q  V  M  A  W  F  R  A  K  T  S  E  A  S  D  V  M  S  F

CTGACATGCTACCACTTTGACCTGCACTGGGAGCACCACAGATGGCCCTTTGCCCCCTGG
 L  T  C  Y  H  F  D  L  H  W  E  H  H  R  W  P  F  A  P  W

                                        C-4 oxygenase Stop
TGGCAGCTGCCCCACTGCCGCCGCCTGTCCGGGCGTGGCCTGGTGCCTGCCTTGGCATGA
 W  Q  L  P  H  C  R  R  L  S  G  R  G  L  V  P  A  L  A  *
```

FIG.1 (cont.)

C-4 oxygenase untranslated region Nos term
CCTGGTCCCTCCGCTGGTGACCCAGCGTCTGCACAAGAGTGTCATGGAGCTCGAATTTCC

CCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTG

CGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAAT

GCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAAT

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end
CTATGTTACTAGATCGGGAATTC

Fig.1 (cont.)

SEQUENCE LISTING

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<170> PatentIn Ver. 2.1

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tttccaacat tttaaatttc actattggct gaatgcttct tctttgagga agaaacaatt 180

cagatggcag aaatgtatca accaatgcat atatacaaat gtacctcttg ttctcaaaac 240

atctatcgga tggttccatt tgctttgtca tccaattagt gactacttta tattattcac 300

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catccgcttc actctttact caaaccaaaa ctcatcaata caaacaagat taaaaacata 1140
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         40                  45                  50

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Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser Asp Ala Ala Arg Pro
     55                  60                  65

gcg cta aag cac gcc tac aaa cct cca gca tct gac gcc aag ggc atc   1434
Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser Asp Ala Lys Gly Ile
 70                  75                  80                  85

acg atg gcg ctg acc atc att ggc acc tgg acc gca gtg ttt tta cac   1482
Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr Ala Val Phe Leu His
                 90                  95                 100

gca ata ttt caa atc agg cta ccg aca tcc atg gac cag ctt cac tgg   1530
Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met Asp Gln Leu His Trp
            105                 110                 115

ttg cct gtg tcc gaa gcc aca gcc cag ctt ttg ggc gga agc agc agc   1578
Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu Gly Gly Ser Ser Ser
        120                 125                 130

cta ctg cac atc gct gca gtc ttc att gta ctt gag ttc ctg tac act   1626
Leu Leu His Ile Ala Ala Val Phe Ile Val Leu Glu Phe Leu Tyr Thr
    135                 140                 145
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ggt cta ttc atc acc aca cat gac gca atg cat ggc acc ata gct ttg    1674
Gly Leu Phe Ile Thr Thr His Asp Ala Met His Gly Thr Ile Ala Leu
150                 155                 160                 165

agg cac agg cag ctc aat gat ctc ctt ggc aac atc tgc ata tca ctg    1722
Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn Ile Cys Ile Ser Leu
                170                 175                 180

tac gcc tgg ttt gac tac agc atg ctg cat cgc aag cac tgg gag cac    1770
Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg Lys His Trp Glu His
            185                 190                 195

cac aac cat act ggc gaa gtg ggg aaa gac cct gac ttc cac aag gga    1818
His Asn His Thr Gly Glu Val Gly Lys Asp Pro Asp Phe His Lys Gly
        200                 205                 210

aat ccc ggc ctt gtc ccc tgg ttc gcc agc ttc atg tcc agc tac atg    1866
Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe Met Ser Ser Tyr Met
    215                 220                 225

tcc ctg tgg cag ttt gcc cgg ctg gca tgg tgg gca gtg gtg atg caa    1914
Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp Ala Val Val Met Gln
230                 235                 240                 245

atg ctg ggg gcg ccc atg gca aat ctc cta gtc ttc atg gct gca gcc    1962
Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val Phe Met Ala Ala Ala
                250                 255                 260

cca atc ttg tca gca ttc cgc ctc ttc tac ttc ggc act tac ctg cca    2010
Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe Gly Thr Tyr Leu Pro
            265                 270                 275

cac aag cct gag cca ggc cct gca gca ggc tct cag gtg atg gcc tgg    2058
His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser Gln Val Met Ala Trp
        280                 285                 290

ttc agg gcc aag aca agt gag gca tct gat gtg atg agt ttc ctg aca    2106
Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val Met Ser Phe Leu Thr
    295                 300                 305

tgc tac cac ttt gac ctg cac tgg gag cac cac aga tgg ccc ttt gcc    2154
Cys Tyr His Phe Asp Leu His Trp Glu His His Arg Trp Pro Phe Ala
310                 315                 320                 325

ccc tgg tgg cag ctg ccc cac tgc cgc cgc ctg tcc ggg cgt ggc ctg    2202
Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu Ser Gly Arg Gly Leu
                330                 335                 340

gtg cct gcc ttg gca tgacctggtc cctccgctgg tgacccagcg tctgcacaag    2257
Val Pro Ala Leu Ala
            345

agtgtcatgg agctcgaatt tccccgatcg ttcaaacatt tggcaataaa gtttcttaag 2317

attgaatcct gttgccggtc ttgcgatgat tatcatataa tttctgttga attacgttaa 2377

gcatgtaata attaacatgt aatgcatgac gttatttatg agatgggttt ttatgattag 2437

agtcccgcaa ttatacattt aatacgcgat agaaaacaaa atatagcgcg caaactagga 2497

taaattatcg cgcgcggtgt catctatgtt actagatcgg gaattc                2543
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<210> 2
<211> 346
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: deduced fusion protein of transit peptide + peptide with beta-carotene C-4 oxygenase activity

<400> 2

Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
1 5 10 15

Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
20 25 30

Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
35 40 45

Asn Gly Gly Arg Val Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser
50 55 60

Asp Ala Ala Arg Pro Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser
65 70 75 80

Asp Ala Lys Gly Ile Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr
85 90 95

Ala Val Phe Leu His Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met
100 105 110

Asp Gln Leu His Trp Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu
115 120 125

Gly Gly Ser Ser Ser Leu Leu His Ile Ala Ala Val Phe Ile Val Leu
130 135 140

Glu Phe Leu Tyr Thr Gly Leu Phe Ile Thr Thr His Asp Ala Met His
145 150 155 160

Gly Thr Ile Ala Leu Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn
165 170 175

Ile Cys Ile Ser Leu Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg
180 185 190

Lys His Trp Glu His His Asn His Thr Gly Glu Val Gly Lys Asp Pro
195 200 205

Asp Phe His Lys Gly Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe
210 215 220

Met Ser Ser Tyr Met Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp
225 230 235 240

Ala Val Val Met Gln Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val
245 250 255

Phe Met Ala Ala Ala Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe
260 265 270

Gly Thr Tyr Leu Pro His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser
275 280 285

Gln Val Met Ala Trp Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val
290 295 300

Met Ser Phe Leu Thr Cys Tyr His Phe Asp Leu His Trp Glu His His
305 310 315 320

Arg Trp Pro Phe Ala Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu
325 330 335

Ser Gly Arg Gly Leu Val Pro Ala Leu Ala
340 345

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01767

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 15/82, C12N 9/02, C12N 9/10, A01H 5/00, C12P 23/00 // (C12N 9/02, C12R 1:89)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, C12P, A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9907867 A1 (CALGENE LLC), 18 February 1999 (18.02.99), see abstract, page 13, lines 15-23, claims --	1-11
X	WO 9806862 A1 (CALGENE, INC.), 19 February 1998 (19.02.98), see page 8. line 9 - page 12, line 15; page 13, line 22 - page 15, line 5 --	1-11
X	Susan Budavari et al "THE MERCK INDEX", twelfth edition", 1996, MERCK & CO., INC. NJ, see entries 890, "Astaxanthin"; 1798, "Canthaxanthin"; 10197, "Xanthophyll". --	8-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 December 2000	20 -12- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Hampus Rystedt/GH Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9818910 A1 (YISSUM RESEARCH AND DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM), 7 May 1998 (07.05.98), see abstract, page 28, line 24 - page 29, line 4	1-4
A		5-11
	--	
A	WO 9613149 A1 (AMOCO CORPORATION), 9 May 1996 (09.05.96)	1-11
	--	
A	EMBL/GenBank/DDBJ databases, accession no. X86782, 1997-11-30, Harker M. et al: "H.pluvialis mRNA for beta-carotene C-4 oxygenase"	4,5
	--	
A	EMBL/GenBank/DDBJ databases, accession no. D45881, 1995-12-29, Kajiwarea S.: "Haematococcus pluvialis mRNA for bet-carotene ketolase, complete cds"	3
	--	
A	EMBL/GenBank/DDBJ databases, accession no. X86783, 1998-06-02, Harker M. et al: "H.pluvialis mRNA for phyteone desaturase"	3
	--	
A	EMBL/GenBank/DDBJ databases, accession no. AF082325, Sun Z. et al: "Haematococcus pluvialis isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase (ipiHp1) mRNA, complete cd, 1998-08-18	3
	--	
X	EMBL/GenBank/DDBJ databases, accession no. AF082326, 1998-08-18, Sun Z. et al: "Haematococcus pluvialis isopenetyl pyrophosphate:dimethylallyl pyrophosphate isomerase (ipiHp2) mRNA, complete cds"	3
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01767

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EMBL/GenBank/DDBJ databases, accession no. AF162276, 1999-09-10, Linden H.: "Haematococcus pluvialis carotenoid hydroxylase mRNA, partial cds" --	3
A	WO 9930701 A1 (ASTACAROTENE), 24 June 1999 (24.06.99), see abstract and claims --	11
A	WO 9837874 A1 (ASTACAROTENE AB), 3 Sept 1998 (03.09.98), see abstract and claims --	11
A	JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY B, Volume 30, 1995, BISWAL, B et al, "Carotenoid catabolism durint leaf senescence and its control by light" page 3 - page 13 -- --------	11

INTERNATIONAL SEARCH REPORT

International application No.
SE00/01767

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

According to Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. The present application relates to five such groups of inventions, namely:

1. A DNA construct encoding an enzyme in the carotenoid biosynthetic pathway and cells expressing the enzyme, according to claims 1-7.
2. Transgenic oilseed plant-produced xanthophyll, according to claim 8.
3. Transgenic oilseed plant-produced canthaxanthin, according to claim 9.
4. Transgenic oilseed plant-produced astaxanthin, according to claim 10.
5. Transgenic oilseed plant-produced astaxanthin esters, according to claim 11.

The feature common to all inventions is the transgenic production of carotenoids in oilseed plants. However, this feature is already known through WO-A1-9806862. The production of different carotenoids, and DNA constructs facilitating the production, is thus not linked by a special technical feature as required by Rule 13.2. As the additional effort of searching inventions 2-5 does not justify an additional search fee, all inventions have been searched.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/SE 00/01767

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9907867 A1	18/02/99	AU 8900298 A	01/03/99
		EP 1002117 A	24/05/00
WO 9806862 A1	19/02/98	AU 4058497 A	06/03/98
		BR 9713462 A	28/03/00
		CN 1227609 A	01/09/99
		EP 0925366 A	30/06/99
WO 9818910 A1	07/05/98	AU 4743697 A	22/05/98
		NO 991996 A	22/06/99
		US 5916791 A	29/06/99
		US 5965795 A	12/10/99
		CN 1247565 A	15/03/00
		EP 0951534 A	27/10/99
		PL 332965 A	25/10/99
WO 9613149 A1	09/05/96	AU 697358 B	01/10/98
		AU 3970195 A	23/05/96
		CA 2203815 A	09/05/96
		CN 1172416 A	04/02/98
		EP 0792352 A	03/09/97
		JP 10509309 T	14/09/98
		NO 971945 A	27/06/97
		NZ 296012 A	28/05/99
		PL 319788 A	01/09/97
		US 5618988 A	08/04/97
WO 9930701 A1	24/06/99	AU 1897299 A	05/07/99
		EP 1049460 A	08/11/00
		NO 20003042 A	14/06/00
		SE 511237 C	30/08/99
		SE 9704693 A	17/06/99
WO 9837874 A1	03/09/98	AU 719090 B	04/05/00
		AU 2796797 A	19/11/97
		AU 6295198 A	18/09/98
		CN 1248912 T	29/03/00
		EP 0898823 A	03/03/99
		EP 0981338 A	01/03/00
		NO 994109 A	27/10/99
		PL 335370 A	25/04/00
		SE 9700708 A	28/08/98